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Cytosolic and Transmembrane Protein Extraction Methods of Breast and Ovarian Cancer Cells: A Comparative Study

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Efficient extraction of proteins is a great challenge for numerous downstream proteomic analyses. During the protein extraction procedure, it is critical to maintain the conformational stability, integrity, as well as higher yield of the protein. To do so, 5-different lysis buffers of Tris and HEPES have been used as the primary buffering reagents with variable compositions at different concentrations and pH using human cancer cells. In this study, different protein lysates of human breast cancer cells T47D and MDA-MB-231 and ovarian cancer cell PA-1 were subjected to run SDS-PAGE for separation of proteins based on their molecular size, followed by Coomassie blue, silver staining, and immunoblot assays to compare the extraction yield of total cytoplasmic proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the integral membrane protein, integrin β-1. Our results revealed that Tris-based lysis buffer with 50 mM concentration, pH 7.5, is relatively the efficient and reliable protein extraction method for a wide range of MW subcellular markers, cytoplasmic GAPDH and transmembrane integrin β-1 proteins. We anticipate that this simple and cost-effective protein extraction protocol might be extremely useful across a broad range of subcellular proteins in different biologic samples.

Key Words: SDS-PAGE, Coomassie blue, silver staining, immunoblot

INTRODUCTION

Proteins are one of the major classes of essential biological macromolecules that are vital for many fundamental processes of an organism. Cellular or subcellular localization of the protein of interest and the protein extraction yield is of great importance to better understand the interactions of protein molecules and overall protein functions. There are several methods available for extraction of enriched proteins from different cellular fractions, such as cytosol and membrane-bound proteins required for several downstream applications. Cytosolic proteins include large complexes of enzymes that are responsible for several metabolic pathways, protein biosynthesis, and other important cell-signaling processes. Indeed, nuclear or cytoplasmic protein extracts are important for various applications, including RNA binding, *in vitro* transcription, mRNA splicing, or gene-expression studies.[1–4] The nuclear proteins are organized into complex regulatory networks and perform varied cellular functions. Recent studies have reported that in all human proteins, membrane proteins comprise ~25% of the total protein[5] and are categorized on the basis of their interaction with the cell membrane, namely, the peripheral membrane proteins that bind noncovalently with the membrane and the integral membrane proteins that bind firmly through hydrophobic interactions.[6] Integral membrane proteins include the cell-surface receptors, signal transducers, metabolite transporters, and membrane channels. In particular, membrane protein integrin subfamilies are cell-adhesion receptors that recognize multiple ligands, such as collagen, fibronectin, laminin, VCAM-1, epiligrin, and invasin, and mediate cell–cell and cell–extracellular matrix interactions through various intra- and intercellular signaling pathways.[7–9] These proteins are important targets of >50% of all modern medicinal drugs. Hence, protein extraction efficiency is a prerequisite for several downstream applications.

Protein extraction involves disruption of the phospholipid bilayer of a cell membrane without affecting the functional activity of the protein structure. Application of detergent, low ionic salt (salting out), shearing force, and rapid pressure change is a critical parameter for enrichment of protein extractions.[10] Certain parameters are very

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Abbreviations: GAPDH=glyceraldehyde-3-phosphate dehydrogenase, M1–M5=protein extraction methods 1–5, TBS=Tris-buffered saline

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crucial for higher yield, as well as improving the stability and integrity of protein molecules, including type of buffers and the concentrations of individual components, as well as the pH of the buffer.[11, 12] In particular, Tris buffers (pH 6.5–9.5), as well as HEPES or phosphate buffers, are widely used as a result of their ability to maintain the conformational integrity of proteins and high solubility.[13–15] Phosphate buffers improve the thermal stability in solutions containing NaCl,[16] delay denaturation of proteins,[17] or stabilize the protein during long-term storage.[18, 19] Ikeuchi *et al.*[17] had reported that sodium dihydrogen phosphate buffer slowed down the denaturation of proteins, which enables the retainment of the functional conformation of the proteins. Sodium fluoride is commonly used as an inhibitor of serine/threonine and acidic phosphatase. Detergent is another significant element needed for extractions of proteins.[11] In addition, extractions are performed at 4°C to avoid denaturation of proteins.

In this study, 5 commonly used protein lysis buffers were studied to determine the efficiency of whole-cell protein extraction from different types of cancer cells, such as human breast cancer cells T47D and MDA-MB-231 and ovarian cancer cell PA-1. These cells represent a good model for cancer research, commonly used for both *in vitro* cell culture and *in vivo* tumor xenograft models.[20, 21] The buffer compositions are listed in **Table 1**. To validate the efficiency of protein extractions, the total protein lysates were subjected to run in SDS-PAGE for separation of proteins based on their molecular sizes, followed by Coomassie blue and highly sensitive silver staining, which are the usually adopted techniques used in molecular biology.[22, 23] We also performed immunoblot analysis using target gene-specific antibodies to identify the low MW cytosolic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and relatively high MW membrane protein integrin β-1 expression in a panel of cancer cell lines. Our studies revealed that Tris-based lysis buffer with 50 mM concentration, pH 7.5, comparatively has better protein extraction efficiency for both cytoplasmic and transmembrane proteins.

TABLE 1

Summary of the compositions of different lysis buffers

Lysis buffer	Composition
M1	10 mM Tris-HCl, pH 7.6
	50 mM NaCl
	50 mM NaF
	1% Triton X-100
	Protease inhibitor
	1 mM EDTA
M2	50 mM Tris, pH 7.5
	150 mM NaCl
	1% SDS
	1% Triton X-100
	Protease inhibitor
M3	62.5 mM Tris-HCl, pH 6.8
	1% β-Mercaptoethanol
	2% SDS
	0.01% Bromophenol blue
	Protease inhibitor
	6% Glycerol
M4	50 mM HEPES, pH 7.4
	150 mM NaCl
	100 mM NaF
	1% Triton X-100
	Protease inhibitor
	10% Glycerol
	1 mM EDTA
	1.5 mM $MgCl_2$
M5	20 mM NaH_2PO_4
	250 mM NaCl
	0.1% SDS
	1% Triton X-100
	Protease inhibitor

MATERIALS AND METHODS

Cell culture and maintenance

T47D and MDA MB-231 cells were obtained from the National Centre for Cell Science, Pune and PA-1 cells were a kind gift from Dr. Sib Sankar Roy (Indian Institute of Cell Biology, Kolkata, India). Cells were routinely maintained in media supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 in a humidified incubator. The cell-culture medium was changed every alternate day or trypsinized for subculture after the cells achieved 80% confluency.

Antibodies and reagents

GAPDH (D4C6R) and integrin β-1 (D2E5) primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Thermo Fisher Scientific (Novex; Waltham, MA, USA). Protease inhibitor cocktail was from MilliporeSigma (Burlington, MA, USA; Cat. No. 539131). Prestained protein ladder, silver-staining kit, Tris base, sodium dihydrogen phosphate, HEPES, glycerol, and EDTA were purchased from HiMedia (Mumbai, India). Unless otherwise indicated, all reagents were obtained from MilliporeSigma.

Extractions of proteins

Cells were cultured in a 60 × 15 mm adherent cell-culture dish in complete growth medium. Cells (1×10^6) were

seeded in 5 dishes of 60 × 15 mm for each protein extraction method (M1–M5). After 2 cells were washed twice with cold PBS, and then, a respective lysis buffer (100 μl) was added in each of the plates, scraped, and incubated for 20 min. Next, the lysed cells were collected in 1.5 ml centrifuge tubes and sonicated for 3 s × 10 amplitude at 4°C, followed by centrifugation at 8000 rpm at 4°C for 20 min. The supernatants were collected, and the total protein lysate from each dish was processed for further analysis to examine the protein extraction efficiency.

SDS-PAGE

Total denatured cell lysates from each dish were subjected to run an SDS-PAGE gel (10% resolving gel and 5% stacking gel) with a dimension of 8.6 × 6.7 cm area and 1 mm thickness, as mentioned in Pal *et al.*[24] and Goh *et al.*[25] Finally, the resolved SDS-PAGE gel was stained with Coomassie brilliant blue to visualize the separation of bands and their intensity for verification of protein extraction efficiency by 5 different methods.

Silver staining of gels

Silver staining of the SDS PAGE gel was performed, as described in Merril and Goldman,[26] with modifications. The gel was fixed in 40% of methanol and 10% of acetic acid with moderate shaking for 1 h, followed by incubation with 10% ethanol and 5% acetic acid for 15 min. Then, the gel was washed twice with Milli-Q water for 15 min each and soaked in fix/sensitizing solution (HiMedia) for 5 min, washed in 30% ethanol for 20 min, and then washed in Milli-Q water for 20 min. After that, the gel was incubated 1 min in sensitizing solution and washed with Milli-Q water, 2 times for 1 min each, followed by 20 min incubation with cold silver-staining solution for 20 min with constant moderate shaking in the dark. The gel was washed for 1 min with Milli-Q water, followed by development of the gel until dark brown bands of protein appeared. After protein bands reached a desired intensity, the reaction was stopped by pouring off developing solution and replacing with 5% acetic acid for 5 min and stored in 1% acetic acid.

Immunoblotting

Immunoblot assay was performed, as described in Li and Benghezal,[27] Jietal et al.,[28] and Zhu et al.[29] For this assay, the resolved proteins in SDS-PAGE were transferred onto an Immobilon PVDF membrane (MilliporeSigma) using transfer buffer containing 192 mM glycine, 25 mM Tris-HCl, pH 8.3, 20% v/v methanol, and 0.02% SDS at 50 V power supply for 1 h. The blot was washed 3 times with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 min each, followed by incubation with blocking buffer (5% nonfat dry milk, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) at room temperature for 1 h on a rocker. After washing 3 times with TBS, the membrane was incubated overnight with the primary antibody at 4°C at the following dilutions: anti-GAPDH mAb 1:1000 and anti-integrin β-1 mAb 1:1000. After washing 3 times (5 min each) with TBS-Tween-20 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated respective secondary antibody at a dilution of 1:5000. Excess secondary antibody was washed off 3 times with TBS-Tween-20 and processed for development of the image with the ECL kit, as recommended by GE Healthcare (Chicago, IL, USA). Densitometric analysis of the Western blotting was performed with an image-scanning device (ChemiDoc; Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

Mammalian adherent epithelial cancer cells T47D, MDA-MB-231, and PA-1 were used to check the protein extraction efficiency using 5 different lysis buffers. It is evident that normal cells stop growing when they confluence as a result of contact inhibition, and therefore, it takes longer time to recover when reseeded for further, subsequent experiments. Whereas cancer cells can grow, continuing even after reaching confluence, they usually worsen after ~2 doublings. Therefore, it is very much needed to subculture the cancer cells in the log phase before they confluence. In our study, during this distinct growth phase of the cells, we captured the microscopic images to observe the morphologic shape pattern in different cells, as represented in **Fig. 1*A*** for T47D cells, **Fig. 2*A*** for MDA-MB-231 cells, and **Fig. 3*A*** for PA-1 cells. Then, we were interested in checking the protein extraction efficacy by 1-dimensional SDS-PAGE, followed by Coomassie brilliant blue staining, using total protein lysates of T47D, MDA-MB-231, and PA-1 cells, as shown in Figs. 1*B*, 2*B*, and 3*B*, respectively. As silver staining is much more sensitive than Coomassie blue staining, we also performed silver staining after running SDS-PAGE gels using the proteins extracted by 5 different protein extraction methods. The extracted protein bands in SDS-PAGE, identified by Coomassie blue, as well as silver staining, show that lysis buffer 2, with Tris base at pH 7.5, has higher protein extraction efficiency based on its higher intensity of bands compared with other lysis buffers when the whole-cell lysates from each dish with same number of seeded cells were resolved in SDS-PAGE.

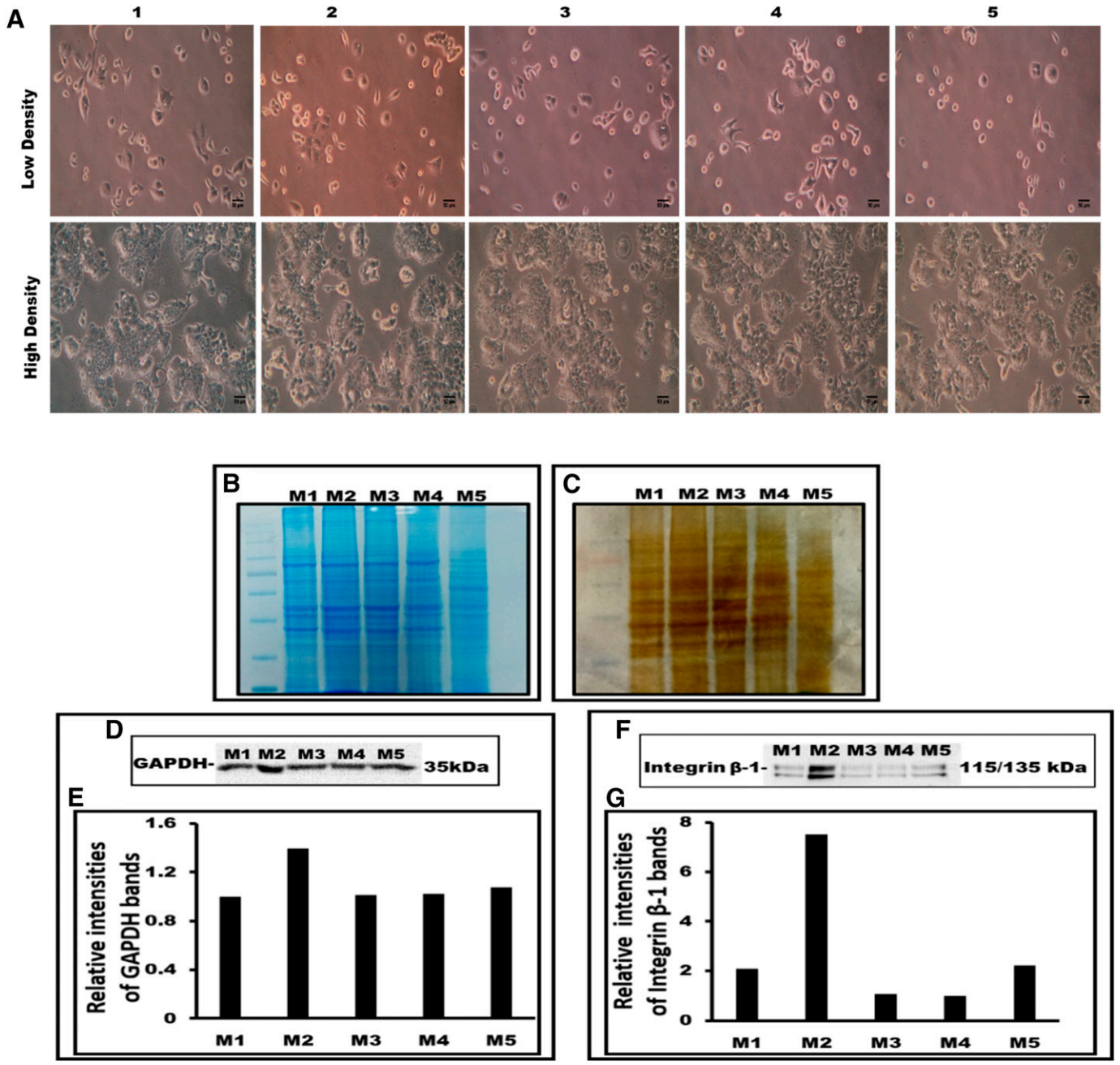

FIGURE 1

Comparative study on protein extractions from T47D cells using 5-different lysis buffers (M1–M5). *A*) Five sets of cancer cells were cultured for extraction of proteins using different lysis buffers. Phase-contrast microscopic images showing the confluency of cells at different days. Images were taken for d 0 and 2. Original scale bars, 50 μm. *B, C*) Coomassie blue (*B*) and (*C*) silver staining after running the SDS-PAGE gel electrophoresis. *D, E*) Immunoblot result (*D*) of protein lysates against GAPDH antibody and their relative intensity (*E*) of protein bands. *F, G*) Immunoblot result (*F*) of protein lysates against integrin β-1 antibody and their relative intensity (*G*) of protein bands.

Next, we were interested in checking the extraction of cytosolic (GAPDH, Molecular mass 37 kDa) and membrane (integrin β-1, MW 115/135 kDa) protein expressions by immunoblot analysis. Our results show that lysis buffer 2 has higher protein extraction efficiency with prominent expressions of the cytosolic protein GAPDH in T47D cells when compared with the other lysis buffers, as represented by the immunoblot results and the corresponding histogram depicting the relative intensities of the bands (Fig. 1*D, E*). In MDA-MB-231 cells, the lysis buffers 2 (M2) and 5 (M5) showed comparable protein extraction efficiency of GAPDH, as

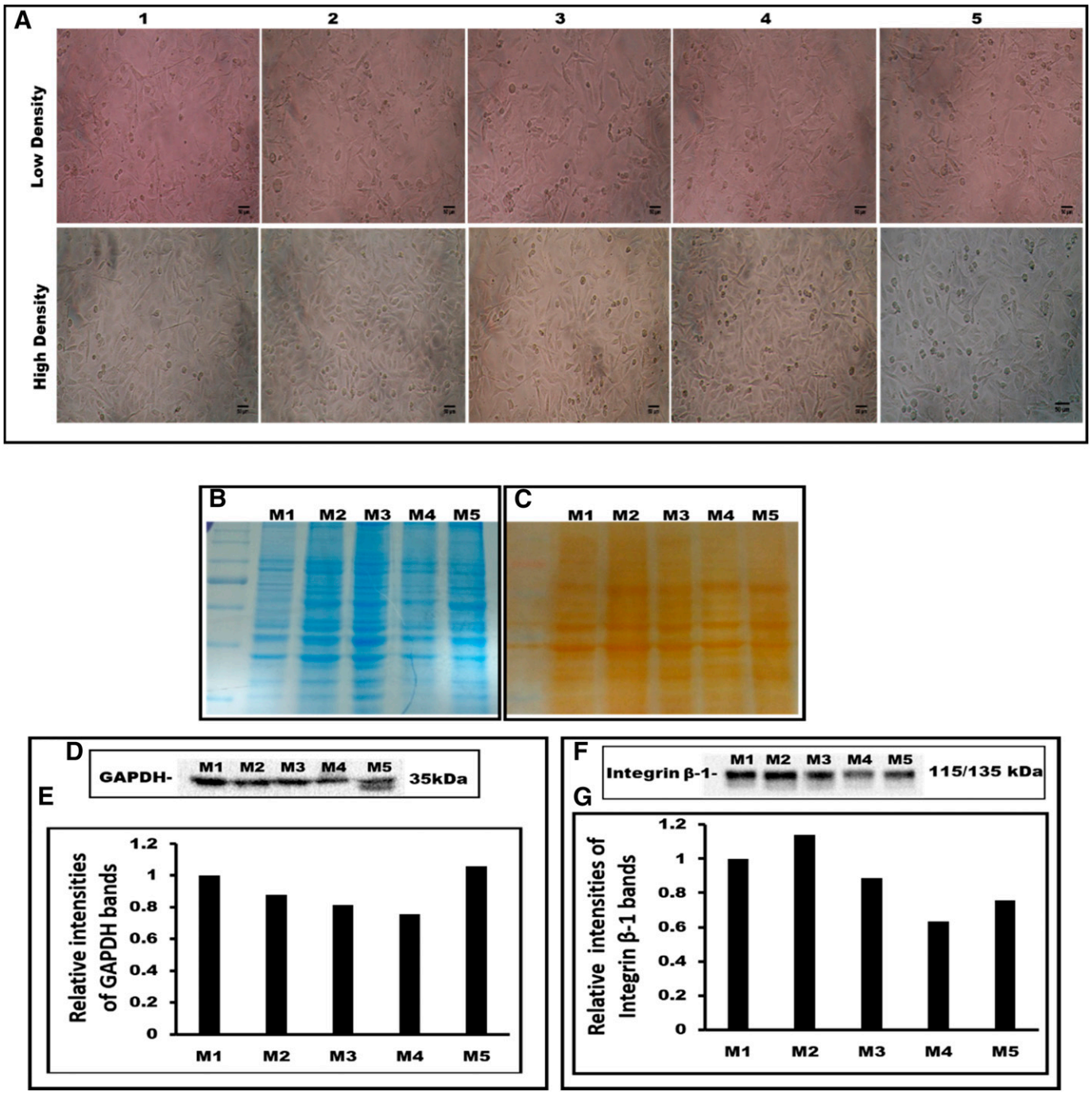

FIGURE 2

Comparative study on protein extractions from MDA-MB-231 cells using 5-different lysis buffers (M1–M5). *A*) Five sets of cancer cells were cultured for extraction of proteins using different lysis buffers. Phase-contrast microscopic images showing the confluency of cells at different days. Images were taken for d 0 and 2. Original scale bars, 50 μm. *B, C*) Coomassie blue (*B*) and silver (*C*) staining after running the SDS-PAGE gel electrophoresis. *D, E*) Immunoblot result of protein lysates against GAPDH antibody and their relative intensity of protein bands. *F, G*) Immunoblot result of protein lysates against integrin β-1 antibody and their relative intensity of protein bands.

shown in Fig. 2*D, E*. When we checked in PA-1 cells, lysis buffers 1 and 2 showed sharply higher protein extraction efficiency based on their intensities of protein bands of GAPDH (Fig. 3*D, E*). Overall, our results suggest that lysis buffer M2 has higher protein extraction efficiency in a cell type-specific manner.

After verification of extraction efficiency of cytosolic proteins using different lysis buffers, we were

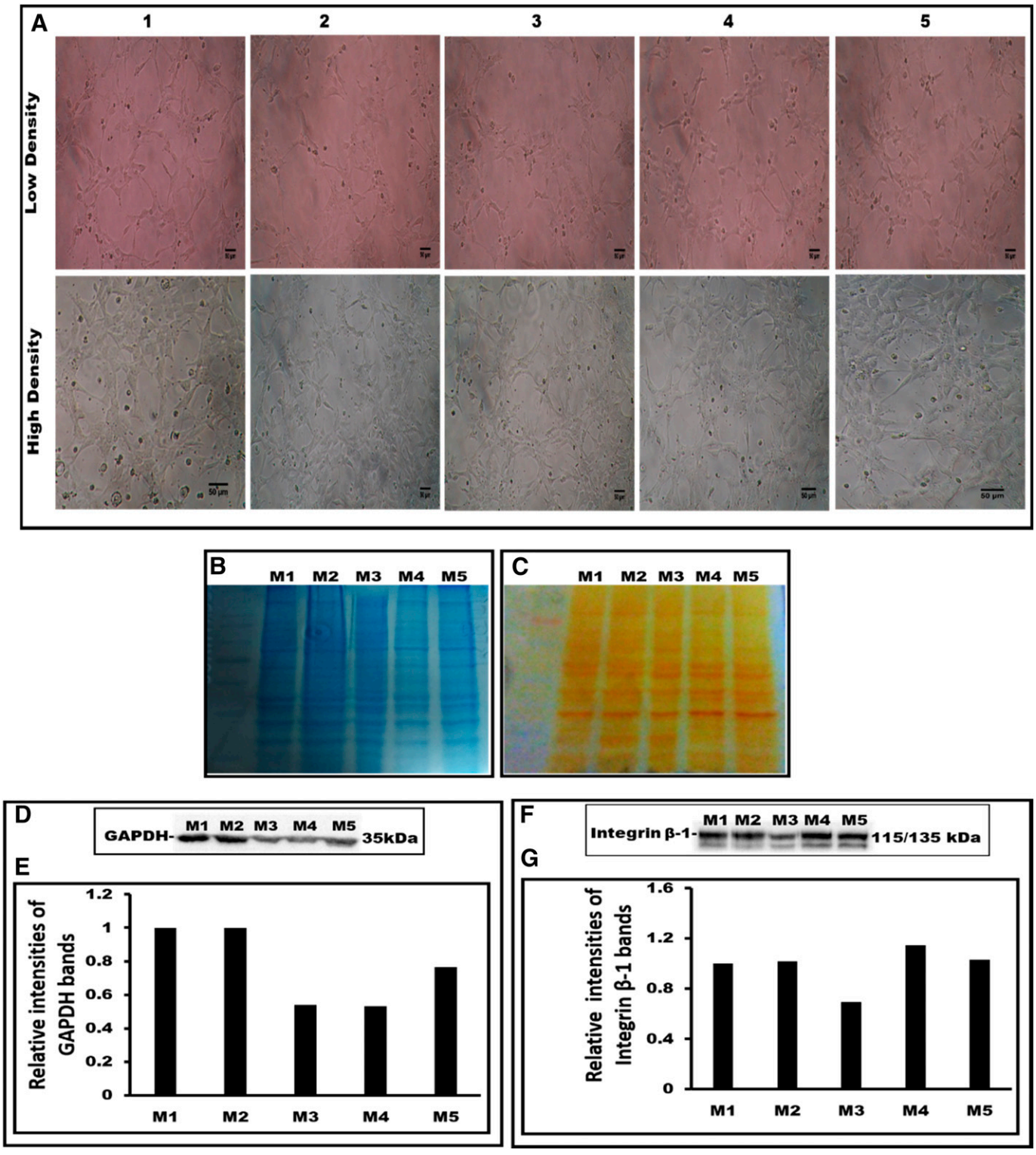

FIGURE 3

Comparative study on protein extractions from PA-1 cells using 5-different lysis buffers (M1–M5). *A*) Five sets of cancer cells were cultured for extraction of proteins using different lysis buffers. Phase-contrast microscopic images showing the confluency of cells at different days. Images were taken for d 0 and 2. Original scale bars, 50 μm. *B, C*) Coomassie blue (*B*) and silver (*C*) staining after running the SDS-PAGE gel electrophoresis. *D, E*) Immunoblot result (*D*) of protein lysates against GAPDH antibody and their relative intensity (*E*) of protein bands. *F, G*) Immunoblot result (*F*) of protein lysates against integrin β-1 antibody and their relative intensity (*G*) of protein bands.

interested in further checking the expression levels of membrane proteins. Transmembrane receptors (integrins) facilitate cell–extracellular matrix adhesion and activate signal-transduction pathways for cell-cycle regulation, proliferation, and migration of cells upon ligand binding. In our study, we found that membrane protein integrin β-1 extraction efficiency is higher in lysis buffer 2, as shown in T47D (Fig. 1*F, G*) and MDA-MB-231 cells (Fig. 2*F, G*). In particular, our results revealed that lysis buffer 2 has a several-fold higher transmembrane protein extraction efficiency than other lysis buffers in T47D cells (Fig. 1*F, G*). In PA-1 cells, all of the lysis buffers show comparable protein extraction efficiency (Fig. 3*F, G*), suggesting that the extraction of membrane proteins by different lysis buffers is a cell type-specific manner, and variation might be a result of the complexities of the membrane proteins located in the cells.

Functional protein enrichment depends on the physiologic pH that determines the structural integrity of proteins,[11] and a slight change in pH may cause a protonation or deprotonation of the amino acid residues, leading to significant loss in the functional activity of the protein. Tris buffers are best known for their effectiveness as physiologic buffers and the structure-stabilizing ability.[13] Although Tris base comprises the buffer compositions of 3 lysis buffers (M1–M3), lysis buffer 2 (M2), at pH 7.5 with a concentration of 50 mM Tris, might be compatible for the maintenance of the structural integrity of the proteins. The higher extraction efficiency of GAPDH proteins in the cancer cells may be a result of the maintenance of thermal stability of phosphate buffer in the presence of NaCl, possibly by phosphate ion binding to the protein.[11, 16]

Overall, enrichment of both cytosolic and membrane proteins from 3 cancer cells using lysis buffer 2 may be the result of the combination effect of 50 mM Tris, pH 7.5, 150 mM NaCl, 1% SDS, and 1% Triton X-100 in the presence of protease inhibitors. In summary, protein extraction methods with lysis buffer 2 (M2) provide the optimal protocol for higher efficiency and feasibility for both the cytosolic protein GAPDH and the transmembrane integrin β-1 protein in different types of cancer cells.

CONCLUSION

Taken together, our results show that the Tris buffer (50 mM) is the optimal lysis buffer for the high-yielding protein extraction method for a wide range of molecular mass of subcellular markers, cytosolic GAPDH (37 kDa) and membrane-bound integrin β-1 (115/135 kDa) proteins. We expect that this buffer composition might be useful for extraction of other cytosolic, as well as membrane, proteins for several downstream applications.

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